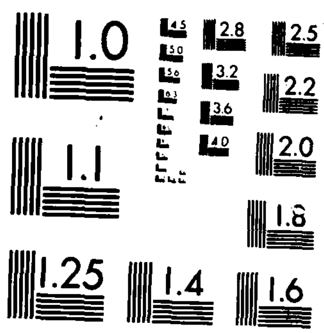
AD-A165 440 VIABILITY OF STORED RABBIT ERYTHROCYTES CARRYING NUMBER 1/1
C3C(U) MASSACHUSETTS UNIV MEDICAL SCHOOL WORCESTER
I O SZYMANSKI 31 MAY 84 DAMD17-82-C-2150

UNCLASSIFIED F/G 6/16 NL



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS 1963-A

(12)

AD ____________

AD-A165 440

Viability of Stored Rabbit Erythrocytes
Carrying No C3c

Annual and Final Report

Irma O. Szymanski, M.D.

May 31, 1984

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2150

University of Massachusetts Medical Center
Worcester, Massachusetts 01605

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents

86 7

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION University of Massachusetts Medical Center 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) Worcester, Massachusetts 01605	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-82-C-2150
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62772A	3S1-62772A874	AA	277

11. TITLE (Include Security Classification)

Viability of Stored Rabbit Erythrocytes Carrying No. C3c

12. PERSONAL AUTHOR(S)

Irma O. Szymanski, M.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual & Final Report	FROM 82/5/15 TO 84/4/30	1984 May 31	27

16. SUPPLEMENTARY NOTATION

Annual cover period (83 Jan 28 through 84 April 30).

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Animal Model to study RBC Preservation, ATP, 2,3-DPG, red cell bound C3, RBC viability, 24 hour survival
06	05		
06	01		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The purpose of these studies was to 1) explore the usefulness of a rabbit model to quantitate RBC preservation injury in vivo and to 2) evaluate the importance of RBC-bound C3 in the preservation injury.

Several technical aspects relating to homologous transfusions of rabbit RBC were investigated during this study period. These included the method of Cr-51 labeling of the preserved RBC and the comparison of single or double labeling technics for assessing RBC viability. Preliminary data obtained with double labeling method indicated that ^{99m}Tc might be a useful label for fresh autologous RBC.

The biological data revealed that ADSOL additive improved the survival of RBC in the initial storage period, but that the viability was not maintained during prolonged

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code) 301/663-7325 — 22c. OFFICE SYMBOL SGRD-RMS

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued)

storage. Destruction of the complement from plasma of the donor units by heat-treatment (to avoid C3 accumulation to RBC membrane) appeared to have adverse effects on RBC survival. It was thought that heat-treatment produced immune complexes. Additional studies ae required to evaluate the role of bound C3 in the preservation injury.

AD ______________________

Viability of Stored Rabbit Erythrocytes Carrying No C3c

Annual and Final Report

Irma O. Szymanski, M.D.

May 31, 1984

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2150

University of Massachusetts Medical Center
Worcester, Massachusetts 01605

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited.

SUMMARY

We have explored the utility of the rabbit model to resolve the question whether C3 bound to stored RBC constitutes a biologically significant preservation injury. Thus, we have worked toward developing a suitable technic to perform RBC survival studies in rabbits. When the technic is perfected, we hope to show that the rabbit model is a suitable substitute for the human model in evaluating RBC preservation in vivo and in answering specific questions about preservation such as the role of bound C3 in the storage lesion.

During this reporting period we have further perfected the RBC survival method in rabbits to evaluate the quality of RBC preservation. We have learned that the labeling of rabbit RBC must be done without a wash procedure since a wash with low pH saline can further damage preserved RBC. It should be noted that the pH in commercial saline is often very low. Our results also showed that the single ^{51}Cr label technic to measure preservation injury in vivo produced only slightly higher 24 hour survival values than the double ^{51}Cr label method. However, we feel that the double label method is scientifically more sound and should be employed in further studies. In order to reduce the variability of the experimental results in the double ^{51}Cr method, we like to use ^{99m}Tc to label fresh autologous RBC. We have experimented with ^{99m}Tc label and found that to be suitable for measurement of RBC mass in rabbits.

We have found that in the rabbit model, addition of Adsol preservative to blood improves the RBC viability over the viability of RBC stored in CPD plasma. In this way the rabbit model is similar to the human model.

Storage of RBC in heated plasma seems to result in decreased cell viability. This could be due to the presence of aggregated material or lack of oxidants in heated CPD plasma. The lack of oxidants is suspected by the results of ^{51}Cr uptake in nonheated and heated plasma. The role of oxidants in cell viability is not known and should be further explored.

Storage of RBC in protein free medium for 35 days decreases both 24 hour survival and long-term survival. It is important to explore whether this is caused by accelerated age-related destruction or by elution of ^{51}Cr label from RBC.

In order to evaluate the biological function of RBC-bound C3, we are planning to store RBC in Adsol containing very small amount of CPD plasma. This would provide a protein-containing medium during storage and prevent C3 uptake by RBC.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

Statement of the Problem

Utilization of human subjects in performing red blood cell (RBC) survival studies to measure the quality of RBC preservation is becoming increasingly difficult. Thus, we have explored whether the rabbit model can be used for this purpose. The rabbit model is advantageous since these animals are relatively easy to handle and they are also inexpensive. The goals of our present work have been as follows:

1. Defining the best technics to perform RBC survival studies in rabbits

2. Evaluating whether the rabbit model is suitable to evaluate the quality of RBC preservation

3. Evaluating whether the accumulation of the third component of complement, C3, to RBC membrane during storage constitutes a biologically significant preservation injury.

Background

RBC that have been stored at $4^{o}C$ either as whole blood or concentrates develop a so-called 'storage lesion' or preservation injury that manifests itself in vivo by the rapid removal of a fraction of RBC (1-3). The nonviable fraction of RBC increases during storage of blood at $4^{o}C$ (4). Although the nonviable RBC are removed rapidly from the circulation, those surviving at 24 hours after transfusion have a potential for normal lifespan (1-3). Although it is not yet possible to measure the preservation injury in vitro, the gradual decline of RBC ATP level during $4^{o}C$ storage is thought to result in the loss of RBC viability (5,6). It has been suggested that the loss of viability may be mediated through decreases in ATP-dependent RBC deformability (7-10). It is undeniable that ATP level is important to donor RBC viability since maintenance of ATP concentration during storage improves viability (7). However, previous reports have shown that correction of RBC ATP concentration by a rejuvenation procedure does not totally normalize RBC viability (11,12). Thus, other factors must also contribute to the development of the preservation injury. We have shown that a C3c-containing fragment of the third component of human complement binds to RBC membrane during storage of blood at $4^{o}C$ (13,14). The longer the storage period at $4^{o}C$, the more C3 becomes bound to RBC. After 21 days of storage, an average of about 44 C3 molecules per cell were bound (14). Studies with RBC stored in either acid-citrate-dextrose (ACD) or citrate-phosphate-dextrose (CPD) anticoagulant at $4^{o}C$ have

shown that the longer the storage period, the larger is the fraction of nonviable RBC that is removed during the initial 24 hour post-transfusion period (4).

We have postulated that coating of stored RBC with C3 might contribute to the loss of their viability. Experiments in rabbits have shown that transfusions of RBC coated in vitro with the third component of complement in the absence of antibodies produced temporary sequestration of the complement coated RBC on the Kuppfer cells (15). Following cleavage of the C3c fragment, most of the RBC returned to the circulation, but some were phagocytized (15). Experiments in vitro have shown that it is difficult to cleave C3c from stored RBC (13). Thus, we hypothesized that even small amounts of C3 on stored RBC could produce a temporary attachment of stored RBC to reticuloendothelial phagocytic cells. This phenomenon could theoretically explain the rapid removal of some stored RBC and normal survival of the remainder.

We have now explored whether the rabbit model could be utilized to resolve the question whether C3 bound to stored RBC constitutes biologically significant preservation injury mediating the loss of nonviabile RBC in vivo. Thus, we have worked toward developing a suitable technic to perform RBC survival studies in rabbits. When the technic is perfected, we hope to show that the rabbit model is a suitable substitute for the human model in evaluating RBC preservation in vivo and in answering specific questions about preservation such as the role of bound C3 in the storage lesion.

Approach to the Problem

Refinement of the Methods to Perform RBC Survival Studies

In order to develop and evaluate an efficient method to quantitate the RBC preservation injury using the rabbit model, it is important to accumulate further experience with the method, become proficient in the various manipulations and to learn what the problems are in this technic. This knowledge would eventually lead to improvements and simplification of the RBC survival technic. It is desirable that the final technic be reproducible and simple and easy to perform by others.

Thus, we have investigated the following technical and scientific parameters:

1. The method of labeling stored RBC with ^{51}Cr
2. Comparison of the % RBC survival measured by double or single label technics
3. Increase in the number of survival studies comparing preservation of RBC in plasma and heated plasma
4. Evaluation of the role of complement in the preservation injury using RBC that were stored in Adsol-saline or in Adsol-plasma

Methods

I. Unit Preparation

a. Collection of Blood used for Homologous Transfusions

Collection of donor blood: A donor rabbit was immobilized on a restraining board. Approximately 5" x 5" area on the anterior chest was shaved using a safety razor contained in a sterile prep kit. Thereafter, the area was washed with Betadine surgical scrub solution (Purdue-Frederich, Norwalk, CT) and the area was draped with sterile towels. Prior to performing the cardiac puncture, the site was once more cleansed with an alcohol swab. The heart cavity was entered with a 50 ml syringe equipped with an 20 gauge needle previously rinsed with 1000 u/ml heparin (L heparin, AlH2 Robins, Richmond, VA). About 40-50 ml of whole blood was drawn whereafter the syringe was removed and the blood was transferred into a 150 ml plastic bag (Fenwal Laboratories) to which the appropriate amount of citrate-phosphate-dextrose (CPD) anticoagulant was added (1.4 ml for each 10 ml of whole blood). Another 50 ml syringe was connected to the needle and a volume of about 30 ml of whole blood was drawn and added to the plastic bag, and the appropriate amount of CPD added. The anticoagulated blood was mixed thoroughly to prevent clotting.

b. <u>Further Processing of the Donor Rabbit Blood</u>

i. <u>Preparation of Plasma Units</u>

Following collection, the blood was centrifuged in a RC-3 centrifuge (Sorvall, Norwalk, CT) at 4C at 4200 rpm for seven minutes. The subsequent procedures were done under a clean air hood. The supernatant plasma was expressed to another plastic bag and divided into two equal parts. The packed RBC (volume about 30 ml) were batch-washed three times (each wash was done with about 167 ml of sterile 0.9% NaCl). After the last wash, most of the supernatant fluid was expressed out of the bag and the RBC were divided into two equal aliquots. Half of the original CPD plasma was added to one aliquot of RBC, and the remaining treated plasma was added to the other aliquot of RBC. After recombining RBC and plasma, the average hematocrit was about 35%.

Thereafter, both units were stored at 4C for up to 21 days.

ii. <u>Preparation of Adsol Units</u>

Donor blood (see "Collection of Donor Blood") was prepared for storage in Adsol (Fenwal Labs) as follows: After washing, RBC were divided into two aliquots. One aliquot received 1/2 volume of Adsol per one volume of RBC and 1/2 volume of autologous plasma. The other aliquot of RBC received 1/2 volume of 0.9% NaCl, pH 7.0 and 1/2 volume of Adsol, pH 7.0. Both the Adsol and 0.9% NaCl solutions were adjusted to pH 7.0 by adding the required volume of 0.2 M Na_2HPO_4. These units were then stored at 4C for up to 35 days.

iii. <u>Preparation of Heat-Inactivated Plasma Units</u>

This treatment of plasma was performed to avoid C3 uptake by RBC during 4C storage. The separated rabbit CPD plasma was incubated at 60C for 120 minutes and centrifuged to clear it of flocculant material. The supernate was recombined with washed rabbit RBC and stored for up to 14 days. The uptake of C3 by RBC was measured by an autoanalyzer hemagglutination method (13) using 1% PVP (polyvinylpyrrolidone, Technicon Corp., Tarrytown, NY) and 1/2500 dilution of goat anti-rabbit C3 (lot #14401E, Cappel Laboratories, Westchester, PA).

c. <u>Quality Control of Units</u>

Blood cultures were done at the time of transfusion by removing a 5 ml aliquot of blood (16). 2.5 ml of blood was added to each of two Bactec vials (Johnston Laboratories, Cockeysville, MD): one for

aerobic and the other for anaerobic culture. The vials were incubated at 35C for up to seven days. The aerobic cultures were placed on a shaker for the first two days. The cultures were read three times during the first two days, thereafter daily. If any of the cultures became positive, the study was discontinued. This service was performed by the Hospital's Clinical Microbiology Laboratory.

II. Double ^{51}Cr Method of Measuring Survival of Stored RBC (20)

a. Labeling of Samples with ^{51}Cr

i. Fresh Blood

2.5 - 3 ml of whole blood was collected from the central ear artery of the rabbit with a heparinized syringe and the contents were evacuated into a 3 ml heparinized test tube containing 100 USP units of Na heparin (B-D, Rutherford, NJ). About 10 uCi of 51 Cr (sodium chromate, sterile, New England Nuclear, Boston, MA) was added into the test tube and the tube was incubated at 22^{o}C for 30 minutes mixing occasionally.

Subsequently, the labeling method was changed to include no wash after RBC labeling.

ii. Stored Blood

Three ml of blood was removed aseptically from the stored unit and placed into a sterile test tube together with about 300-350 uCi 51 Cr. The blood and 51 Cr were incubated at 22^{o}C for 30 minutes mixing occasionally. Then the labelled RBC were washed three times with sterile 0.9% NaCl. In some experiments, the pH of the wash solution was controlled to be either 7.0 or below 5.0. Since it appeared that wash with low pH 0.9% NaCl affected RBC survival adversely, all the latest studies were done without wash of the RBC after labeling.

iii. Loading Syringes

An accurate volume of one ml was drawn through a 20 gauge needle into a tuberculin syringe for injection. A 20 gauge 1 1/2" needle is kept on the syringe for injection. The remainder of the sample was prepared to be counted for radioactivity.

b. Injection of the blood samples into the recipient rabbit and collection of the post-transfusion blood samples

The marginal ear vein of the rabbit was entered with a 23 gauge catheter (Minicath prn, Intermittent injection set, Deseret Co.,

Sandy, Utah). The labeled fresh blood was injected completely. Thereafter, about 5 ml of sterile 0.9% NaCl was injected to clear the catheter of RBC. About 30 minutes later a 2 ml heparizined blood sample was collected from the vein in the other ear. Then, the 1 ml labeled stored blood was injected through the catheter as described above.

Post-transfusion blood samples were collected 3 and 30 minutes after this injection. Subsequent blood samples were collected 24 and 48 hours later and, if possible, 3, 5 and 7 days later.

c. <u>Preparation of the blood samples for counting</u>

A 1/25 dilution was prepared from the labeled, washed samples of the injected fresh and stored blood. A 1 ml aliquot was pipetted into counting tubes in triplicate. The pipetting protocol was changed in all subsequent studies where post-labeling wash procedure was eliminated. In these cases, the 1/25 dilution was centrifuged and 1 ml of the supernatant fluid from this was also pipetted in triplicate for counting.

From the post-transfusion samples, 1 ml aliquots were pipetted. In cases where the injected, labeled RBC had not been washed, post-transfusion plasma samples were also counted (only for the 5 and 30 minutes post-injection samples).

Spun hematocrits were done in duplicate on all the samples: the injected labeled bloods, as well as the post-transfusion samples.

d. Calculations

All counts were corrected for background. In the following, CPM refers to corrected counts.

A = the total net CPM injected in the fresh samples = 25 x CPM (fresh std) or in nonwashed samples:

A = 25 x (CPM (WB, fresh std) - (1 - Hct) x CPM (super, fresh std)

B = the total net CPM injected in the stored sample = 25 x CPM (stored std)

$$\% \text{ uptake} = \frac{\text{CPM (WB,std)} - (1\text{-Hct}) \times \text{CPM(super,std)}}{\text{CPM(WB, std)}}$$

For each post injection sample, we calculated net counts/ml packed RBC as follows:

$$\text{net CPM/cc PC} = \frac{\text{CPM (whole blood)}}{\text{Hct}/100}$$

or when nonwashed, labeled RBC were injected

$$\text{net CPM/cc PC} = \frac{\text{CPM(WB)} - \text{CPM(WB)} \times (1 - \text{Hct})}{\text{Hct}/100}$$

For each sample, this value was coded with the following notation:

CPM(0-f) for the sample obtained 30 minutes after fresh blood injection

CPM(3-st) or CPM(30-std) for the sample obtained 3 or 30 minutes respectively after stored blood injection

CPM(24 hours) in the 24 hour sample post-injection

CPM(48 hours) in the 48 hour sample post-injection, etc.

RBC mass = A/CPM(0-f)

Survival at 0-time =

$$100 \frac{(\text{RBC mass}) \times (\text{CPM}(0\text{-st})-\text{CPM}(0\text{-f}))}{B}$$

% survival at 24 hours =

$$100 \frac{(\text{RBC mass}) \times (\text{CPM}(24 \text{ hours}) - 0.92 \times \text{CPM}(0\text{-f}))}{B}$$

% survival at 48 hours =

$$100 \frac{(\text{RBC mass}) \times (\text{CPM}(48 \text{ hours}) - 0.84 \times \text{CPM}(0\text{-f}))}{B}$$

% survival at 3 days =

$$100 \frac{(\text{RBC mass}) \times (\text{CPM}(3 \text{ days}) - 0.80 \times \text{CPM}(0\text{-f}))}{B}$$

% survival at 5 days =

$$100 \frac{(\text{RBC mass}) \times (\text{CPM}(5 \text{ days}) - 0.66 \times \text{CPM}(0\text{-f}))}{B}$$

% survival at 8 days =

$$100 \frac{(\text{RBC mass}) \times (\text{CPM}(8 \text{ days}) - 0.62 \times \text{CPM}(0\text{-f}))}{B}$$

e. <u>Survival studies performed</u>

Since last annual report, the following number of survival studies were performed:

Fresh RBC = 12 studies
RBC stored in CPD plasma = 54
RBC stored in heated CPD plasma = 29
RBC stored in Adsol and plasma = 27
RBC stored in Adsol and 0.9% NaCl = 27

Results

^{51}Cr Survival Studies using Fresh Autologous Rabbit RBC

The survival curve of fresh autologous rabbit RBC is shown in Figure 1. These studies represent means obtained in 12 rabbits. The recovery at 24 hours was arbitrarily assumed to be 100%. T 1/2 is 13 days, which is almost identical to the value obtained by us previously.

Effects of Low pH Wash on 24 Hour Survival

Table 1

Comparison of the 24-hour survival of RBC washed with 0.9% NaCl, pH about 4.5, to the 24-hour survival of RBC which had not been washed, or were washed with 0.9% NaCl, pH about 7.0. All RBC had been stored for 21 days.

	Washed with 0.9% NaCl pH 4.5	Nonwashed or washed with 0.9% NaCl pH 7.0
24-hour survival mean	51.6	69.1
S.D.	14.8	17.6
N	10	14
t-test	t = 2.45	p 0.005

Labeling Characteristics when No Post-Labeling Wash Procedure Was Involved

Table 2 shows the % Cr uptake by RBC which were labeled in autologous plasma and subsequently not washed, and by stored RBC labeled in the presence and absence of plasma. It was apparent that fresh plasma competed for ^{51}Cr label, decreasing the ^{51}Cr uptake by RBC, whereas heated plasma did not. When the percentage of plasma in the sample was decreased, the percent uptake increased.

Table 2
^{51}Cr uptake by fresh and by preserved RBC

Characteristics of the labeled blood	Number of samples studied	% ^{51}Cr uptake by RBC Mean + S.D.
Fresh blood collected in heparin	60	87.49 ± 3.58
Blood stored in CPD plasma	6	95.3 ± 5.06
Blood stored in heated CPD plasma	6	98.1 ± 0.34
Blood stored in Adsol + plasma	12	97.71 ± 0.905
Blood stored in Adsol + saline	12	98.56 ± 0.524

RBC Mass of Rabbits

Utilizing ^{51}Cr labeling technic as described above, analysis of the data in 60 rabbits revealed that the average RBC mass/kg was 17.745 ml/kg with a standard deviation of 2.85 ml.

Double or Single Label?

Comparison of the 24 hour survival values measured both by the double and single label methods in 20 rabbits is shown in Figure 2. The data for analysis of the single label studies were calculated by assuming that the recovery of the labeled RBC at 3 minutes after transfusion was 100%. The recoveries of Cr labeled RBC at other times were then expressed as a proportion of this value. Figure 2 shows that the 24 hour survival values of stored RBC, whether measured by single or double label methods, were very similar although the results of the double label method were consistently slightly lower. Figure 3 shows the comparison of the average survival values obtained by single and double label methods at 3 minutes, and the 24 hour after transfusion, as well as the % survival value obtained by extrapolation of the survival curve to the y-axis. This latter value is indicative of the total % of the viable RBC in the transfusion.

Figure 4 shows the compiled results of all the 24 hour survival values done using the various storage technics. The 24 hour survival of RBC stored in heated CPD plasma and in CPD plasma for 7 days was 87 $\pm$ 15.3% (n = 10) and 87.0 $\pm$ 11.9% (n = 10) days, respectively.

The mean survival of RBC in heated CPD plasma and in CPD plasma stored for 14 days was 85.3 $\pm$ 6.75 (n = 17) and 90.6 $\pm$ 7.98 (n = 28) respectively. This difference was statistically significant ($t = 2.23$, $DF = 43$, $p < 0.05$). The mean survival of RBC stored in heated CPD plasma and in CPD plasma for 21 days was 58.45 $\pm$ 22.64 (n = 17) and 68.11 $\pm$ 18.92 (n = 29) respectively. Although the mean survival of RBC stored in heated plasma tended to be lower than the mean survival of RBC stored in CPD plasma, these differences were statistically not significant, probably due to the variability in the data.

The mean 24 hour survival of RBC stored in Adsol and plasma or Adsol and saline for 21 days was 81.4 $\pm$ 14.4% (n = 18) and 77.9 $\pm$ 11.89 (n = 18) respectively. These values were not significantly different. The survival of RBC stored in Adsol and plasma for 21 days was significantly higher than the survival of RBC stored in CPD plasma ($t = 2.501$, $DF = 45$, $p < 0.05$).

The mean 24 hour survival of RBC stored in Adsol and plasma or in Adsol and saline for 35 days was 53.2 $\pm$ 12.4% (n = 9) and 38.7 $\pm$ 12.17% (n = 9) respectively. The survival of RBC stored in Adsol and plasma for 35 days was significantly lower than the survival of RBC stored in CPD plasma for 21 days ($t = 2.30$, $DF = 36$, $p < 0.05$).

When the t 1/2 of the survival of stored RBC was calculated, it appeared that on the average, t 1/2 was shortened after prolonged storage. Following storage of RBC in Adsol and plasma for 21 days and 35 days, the mean t 1/2 were 11.7 and 9.2 days respectively. Following storage of RBC in Adsol and saline for 21 days and 35 days, the mean t 1/2 were 9.3 and 5.5 days, respectively. Thus, it appears

that prolonged storage of RBC in the absence of any plasma components decreased the t 1/2 of RBC survival.

Figure 1

The survival of autologous fresh rabbit RBC. Studies were done in six animals and the points shown represent means. The survival at 24 hours was arbitrarily taken as 100%

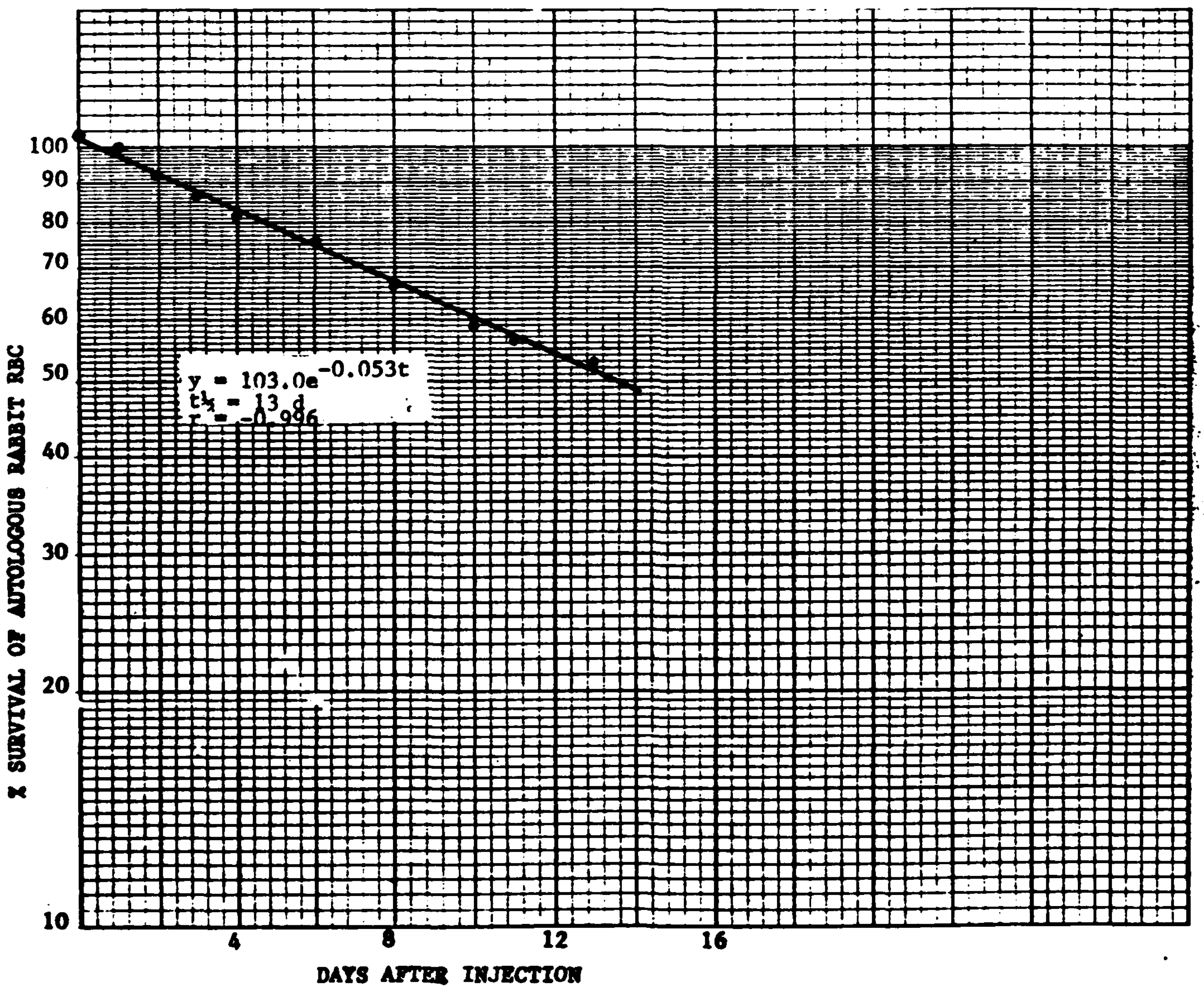


Figure 2

Relationship between the 24 hour survival value obtained by single and double labeling methods

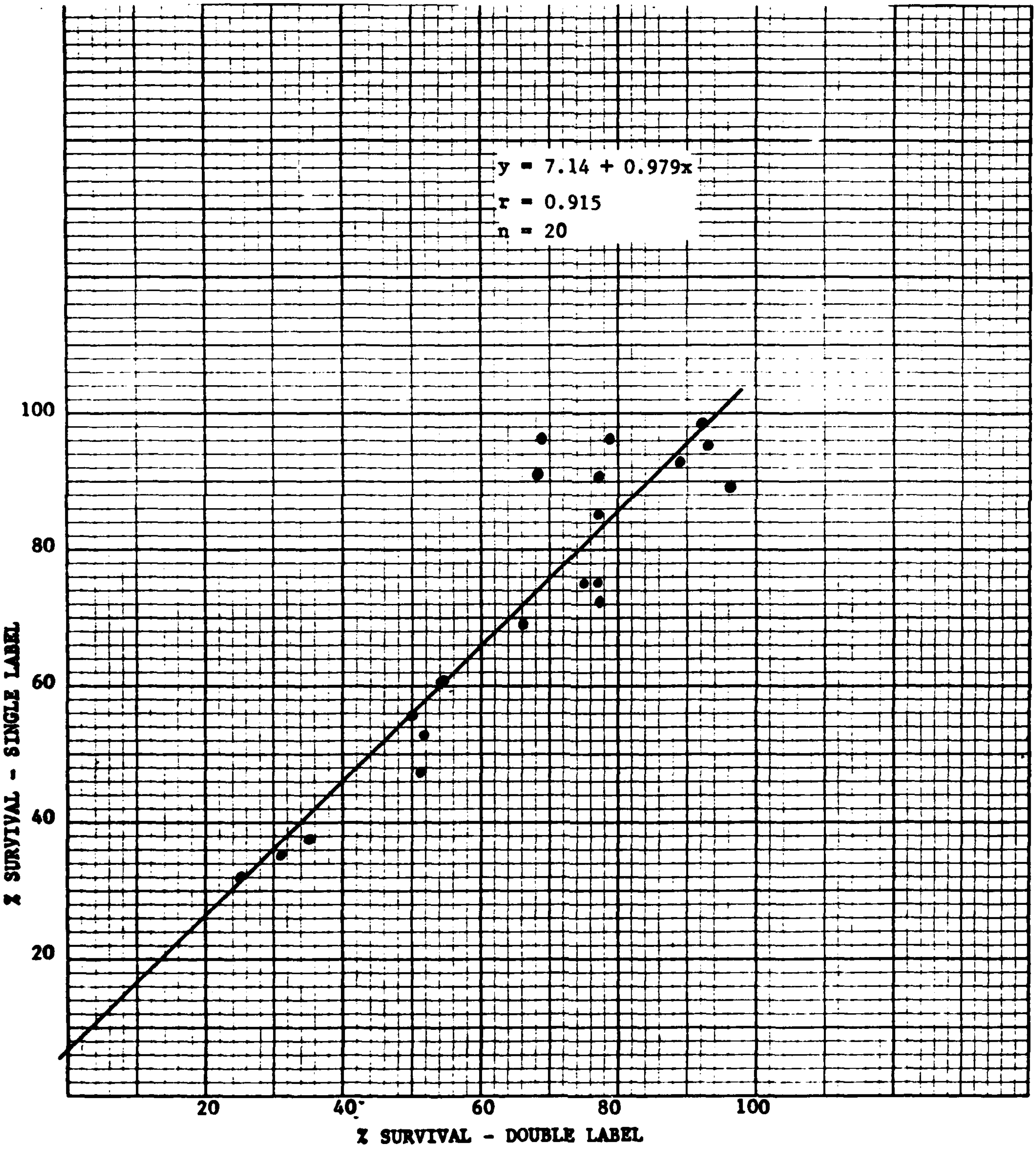


Figure 3

Comparison of the measurement of rabbit RBC survival by the single and double labeling methods at 3 minutes and 24 hours post-transfusion. The % of viable RBC in the unit transfused is also indicated.

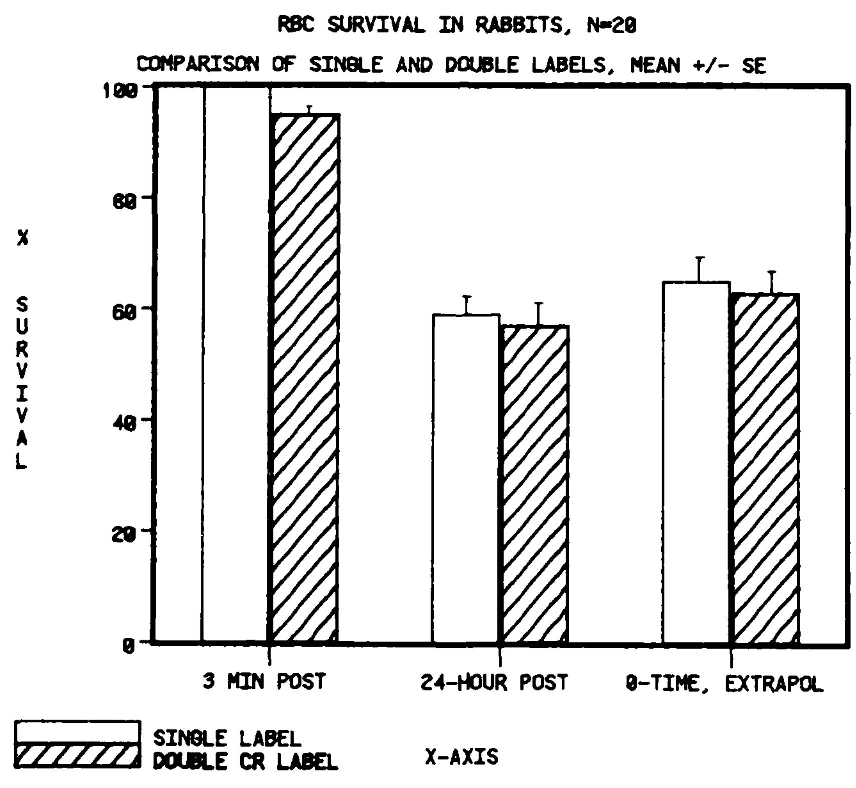
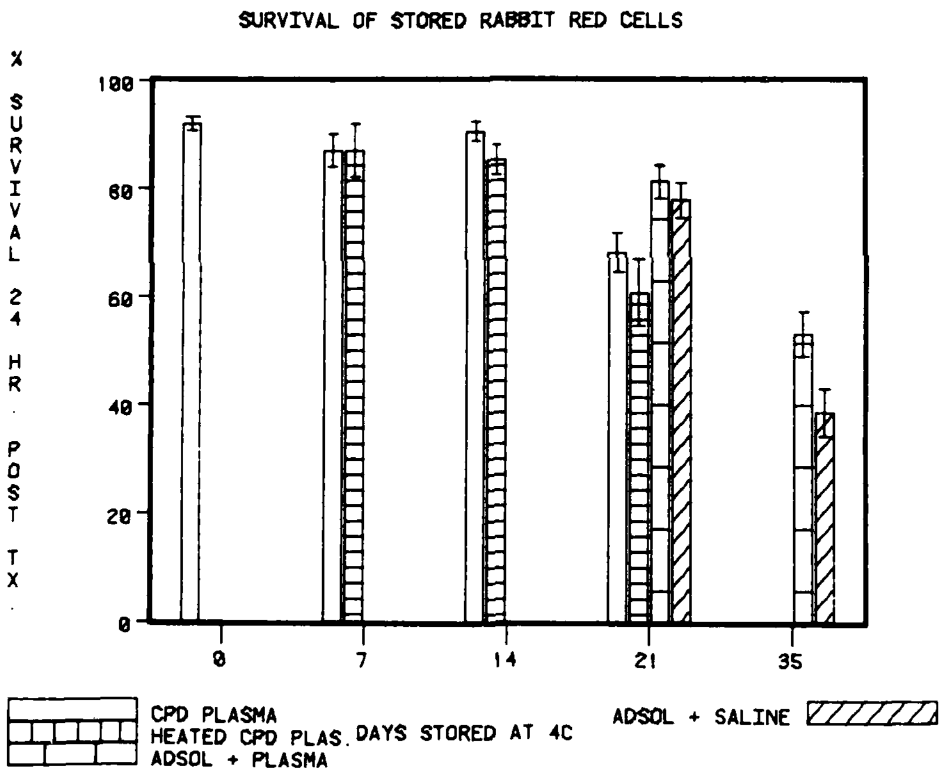


Figure 4

The mean ± SE of the 24-hour survival value of variously preserved rabbit RBC.



Discussion

The data collected during this contract period emphasize several important points. First, the technical details of performing the RBC survival can actually in themselves have detrimental effect on the 24 hour survival value. We observed that if RBC stored at $4^{o}C$ for 21 days, are washed with 0.9% NaCl, pH below 4.5, they have, on the average, significantly lower 24 hour survival values than similarly stored RBC that were either not washed after labeling or were washed with 0.9% NaCl, pH 7.0. When measuring the pH of commercially available 0.9% NaCl, we observed that the pH was often below 5.0. Thus, it appears that in order to get accurate information regarding the quality of RBC preservation, the RBC labeling should not be followed by a washing procedure.

Another interesting feature of these data was that the uptake of 51Cr label was decreased when RBC were suspended in fresh plasma but not when inactivated plasma was used. When RBC were labelled in the presence of stored plasma, the ability of plasma to compete for 51Cr label had diminished. It is known that during the labeling procedure, only hexavalent 51Cr can enter into RBC and label the beta chain of the globin molecule (17-18). When ascorbic acid is added, all 51Cr is converted to trivalent form, which is no more able to label RBC (19). The natural oxidants in plasma are probably res- nsible for lowered 51Cr uptake by RBC when they are suspended in plasma during the labeling. It appears, therefore, that during blood storage the level of these natural oxidants in plasma declines.

There is currently a controversy going on regarding whether a double or single labeling method is necessary to evaluate RBC preservation in vivo. We have decided to use the double label method since if it is well done, the RBC preservation injury can be quantitated more accurately than is possible by the single label method. We were also able to calculate our results as if they were obtained by only the single label method, assuming that the recovery at 3 minutes post-transfusion equalled 100%. Our data show a slightly higher recovery obtained by single label technic, but on the average, these differences were very small. It appeared to us that the results obtained by the single label technic were less variable than those obtained by the double label method. This could relate to technical problems such as inaccurate volume of the injected material, extravasation of the sample during injection, etc. Although the results obtained with the single label method were only slightly higher than with the double label method, I would recommend the use of the double label technic in future RBC survival studies. In order to reduce variability in the results, I would like to use another label than 51Cr to measure RBC mass. This would permit simultaneous injection of labeled fresh autologous and labeled stored RBC. Even if the injection volume would be inaccurate and even if extravasation would occur during injection, comparison of the ratio of the two labels at 15 minute post-transfusion will reveal whether it deviates from the expected ratio of 100% survival. Our preliminary data at the present time show that ^{99m}Tc is an accurate label to measure RBC mass in rabbits. We found the RBC mass in rabbits measured by a 51Cr method to be 17.7 mg/kg.

Our method of evaluating the role of C3 in the preservation injury was to obtain a "unit" of whole blood from a donor rabbit, isolate and wash RBC from it

and divide them into two parts. One part was then stored so that C3 uptake by RBC was inhibited and the other unit was stored similarly but C3 was permitted to accumulate onto RBC membrane. Heating of plasma to 60°C for two hours prevented C3 uptake by RBC. However, survival of RBC stored in the heated plasma was actually decreased. Analyzing the uptake of 51Cr in heated plasma suggested to us that heating depleted naturally occurring oxidants from plasma. This could possibly affect RBC survival. There is also a possibility that the aggregates formed during heating of plasma might combine with the RBC during storage and cause their accelerated destruction after transfusion.

The other approach was to store the washed RBC in Adsol and saline (pH about 7) to prevent C3 uptake in contrast to storage of RBC in Adsol and plasma to permit C3 uptake. However, even in RBC stored in Adsol and plasma we observed less C3 uptake than expected and in some cases no C3 was observed at all. Thus, this method did not turn out to an ideal one to study the specific role of RBC-bound C3. In fact, RBC stored in Adsol without any plasma for 35 days had significantly lower RBC survival than those stored in the Adsol and plasma. It appears, therefore, that some plasma component is required to maintain RBC integrity during storage.

However, our data also show that RBC stored in plasma and Adsol have significantly better 24 hour survival values than RBC stored in CPD plasma alone, indicating that the Adsol improves the RBC survival in rabbit model as it does in human model. This implies that the rabbit model is useful in evaluating the quality of RBC preservation. Our studies with Adsol involved a lot of manipulation and therefore might not be totally comparable to preservation of human RBC by the Adsol technic.

It appears now that we need to investigate whether C3 uptake can be totally prevented by storing packed RBC in Adsol. These RBC would have some plasma proteins in the medium to prevent the storage damage occurring that we observed when they were stored in the absence of plasma proteins. The survival of these RBC should then be compared to the survival of RBC stored in Adsol containing large quantity of plasma, permitting C3 uptake. The fact that t 1/2 of the RBC that had been stored in protein free medium for prolonged periods was decreased is interesting since usually the preservation injury affects only the 24 hour survival value, but not the long-term survival. It is important to determine whether the decrease in t 1/2 indicates rapid elution of Cr label from RBC or decrease of RBC lifespan.

Conclusions

During this reporting period we have further perfected the RBC survival method in rabbits to evaluate the quality of RBC preservtion. The purpose of this work has been to make it possible to use rabbit model instead of human model to evaluate RBC preservation in general and the role of RBC-bound C3 in the preseration injury in particular.

We have learned that the labeling of rabbit RBC must be done without a wash procedure since a wash with low pH saline can further damage preserved RBC. It should be noted that the pH in commercial saline is often very low. Our results also showed that the single 51Cr label technic to measure preservation injury in vivo produced only slightly higher 24 hour survival values than the double 51Cr label method. However, we feel that the double label method is scientifically more sound and should be employed in further studies. In order to reduce the variability of the experimental results in the double 51Cr method, we like to use 99mTc to label fresh autologous RBC. We have experimented with 99mTc label and found that to be suitable for measurement of RBC mass in rabbits.

We have found that in the rabbit model, addition of Adsol preservative to blood improves the RBC viability over the viability of RBC stored in CPD plasma. In this way the rabbit model is similar to the human model.

Storage of RBC in heated plasma seems to result in decreased cell viability. This could be due to the presence of aggregated material or lack of oxidants in heated CPD plasma. The lack of oxidants is suspected by the results of 51Cr uptake in nonheated and heated plasma. The role of oxidants in cell viability is not known and should be further explored.

Storage of RBC in protein free medium for 35 days decreases both 24 hour survival and long-term survival. It is important to explore whether this is caused by accelerated age-related destruction or by elution of 51Cr label from RBC.

In order to evaluate the biological function of RBC-bound C3, we are planning to store RBC in Adsol containing a very small amount of CPD plasma. This would provide a protein-containing medium during storage and prevent C3 uptake by RBC.

RECOMMENDATIONS

It is advantageous to simplify the measurement of RBC survival by using 99mTc as the label for fresh autologous RBC and 51Cr as a label for stored RBC to evaluate the % survival of stored RBC at 24 hours and the rate of destruction thereafter.

In order to validate the rabbit model as a substitute for the human model, we need to evaluate whether optional additives, such as CPDA-1 and CPDA-2 provide an advantage as a storage medium over the CPD-anticoagulant.

To study the biological role of RBC-bound C3 we will evaluate a system that permits storage of RBC in the presence of plasma without resulting in C3 uptake by RBC. We suggest that RBC stored in small amount of CPD plasma and Adsol might be such a system.

REFERENCES

1. Szymanski IO, Valeri CR: Automated differential agglutination technic to measure red cell survival. II. Survival in vivo of preserved red cells. Transfusion 8:74-83, 1968.

2. Valeri CR: Viability and function of preserved red cells. NEJM 284:81-88, 1971.

3. Mollison PL: Blood transfusion in clinical medicine. Blackwell Scientific Publication, Oxford, p.139, 1983.

4. Valeri CR, Szymanski IO, Zaroulis CG: 24 hour survival of ACD- and CPD-stored red cells. Vox Sang. 22:289-308, 1972.

5. Nakao K, Wada T, Kamiyama T: A direct relationship between adenosine biphosphate level and in vivo viability of erythrocytes. Nature 194:877-878, 1962.

6. Akerblom O, Verdier CH, Finnson M, Garby L, Johansson SGO: Further studies on the effect of adenine in blood preservation. Transfusion 7:1-9, 1967.

7. Simon ER: Red cell preservation: Further studies with adenine. Blood 20:485-491, 1962.

8. Weed RI, LaCelle PL, Merrill EW: Metabolic dependence of red cell deformability. J. Clin. Invest. 48:795-809, 1969.

9. Haradin AR, Weed RI, Reed CF: Changes in physical properties of stored erythrocytes. Relationship to survival in vivo. Transfusion 9:229-237, 1969.

10. Moore GL, Antonoff RS, Rau DC: The changes in the erythrocyte membrane during cold storage of whole blood. Vox Sang. 22:481-487, 1972.

11. Valeri CR, Zaroulis CG: Rejuvenation and freezing of outdated stored human red cells. NEJM 287:1307-1313, 1972.

12. Mollison PL, Robinson MA: Observations on the effects of purine nucleosides on red cell preservation. Brit. J. Haematol. 5:331-343, 1959.

13. Szymanski IO, Odgren PR: Studies on the preservation of red blood cells. Attachment of the third component of human complement to erythrocytes during storage at 4C. Vox Sang. 36:213-224, 1979.

14. Szymanski IO, Swanton R, Odgren PR: Quantitation of the third component of complement, C3, on stored erythrocytes. Transfusion 24:194-197, 1984.

15. Brown DL, Lachmann PJ, Dacie JV: The in vivo behaviour of

complement-coated red cells. Studies in C6-deficient, C3 depleted and normal rabbits. Clin. Exp. Immunol. 7:401-421, 1970.

16. DeLand F, Wagner HN: Automated radiometric detection of bacterial growth in blood cultures. J. Lab. Clin. Med. 75:529, 1970.

17. Valeri CR: Observations on the chromium labelling of ACD-stored and previously frozen red cells. Transfusion 8:210-219, 1968.

18. Pearson HA: The binding of Cr 51 to hemoglobin. II. In vivo elution rates of Cr 51 from HbCC, HbCS and placental red cells. Blood 28:563-567, 1966.

19. Cline MJ, Berlin NI: The red cell chromium elution rate in patients with some hematologic diseases. Blood 21:63-69, 1963.

DISTRIBUTION LIST

4 copies	Commander Letterman Army Institute of Research (LAIR), Bldg. 1110 ATTN: SGRD-ULZ-RC Presidio of San Francisco, CA 94129-6815
4 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, Maryland 21701-5012
12 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

END
FILMED
4-86
DTIC